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Note

High-performance liquid chromatography of triterpene saponins

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Triterpene saponins, glycosides of potential commercial interest, are composed of a tetra- or pentacyclic triterpene aglycone substituted with one or two sugar side chains. These compounds possess (among others) anti-inflammatory, hypocholesterolemic, antifungal, contraceptive and foaming properties[1] that are useful in medicine, agriculture and industry. Triterpene saponins are commonly distributed in most plant taxonomic groups and are present in numerous commercially important crops, such as sugar beets, alfalfa, spinach and soybeans[2].

Saponin isolation has been carried out by time-consuming techniques, such as thin-layer chromatography and low-pressure open column chromatography. Gas–liquid chromatography might be used but requires derivatization and high temperatures. In contrast, high-performance liquid chromatography (HPLC) generally has high resolving power and speed, and may be used for the separation of underivatized saponins at room temperature.

Analyses of triterpene saponins by HPLC have been reported for derivatized ginsenosides[3], diene-transformed saikosaponins[4], underivatized glycyrrhizic acid[5], (which differs from most saponins by its capacity to absorb UV light at 254 nm), and saponins extracted from *Phytolacca dodecandra*[6]. Our goal was to find a method for resolving mixtures of underivatized saponins. Although we have developed improved methods for the separation and the characterization of aglycones from *Chenopodium quinoa* saponins by HPLC[7] and gas chromatography–mass spectrometry (GC–MS)[8], our studies on saponin metabolism and biological properties required a convenient procedure for the analysis and purification of whole saponins.

We report an efficient reversed-phase HPLC (RP-HPLC) procedure to resolve eight underivatized triterpene saponins including two glucuronic acid-containing saponins on a C_{18} column using a 25-min water–acetonitrile gradient in the presence of trifluoroacetic acid (TFA).

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EXPERIMENTAL*

Standards

The triterpene saponins, madecassoside, hederacoside C, asiaticoside, chrysantellin A, echinocystic acid-3-glucoside, α-hederin, gypsogenin-3-glucuronide and β-escin, were obtained from Sarsyntex (Merignac, France). The β-escin contained several minor impurities[9].

Instrumentation

The liquid chromatograph consisted of an automatic sample injector (Waters Assoc., WISP 710), a solvent delivery system (Spectra Physics, SP 8700) and a photodiode array detector (Hewlett-Packard 1040A) connected to a HP-85 computer to monitor chromatographic parameters and process data. Columns (Alltech, 250 × 4.6 mm I.D.) were prepacked with 5-μm C_{18} or with 5-μm cyano-bonded particles; they were protected by 25 × 3.9 mm I.D. C_{18}/Corasil and cyano/Corasil guard columns, respectively.

Chromatographic conditions

Chromatographic runs were carried out with two acetonitrile–water gradient elution systems. Solvents were 100% water (solvent A) and 100% acetonitrile (solvent B). In some experiments, 0.1% (final concentration) TFA was added to solvents A and B. Gradients used were linear from 0 to 80% acetonitrile in water over 25 min plus a plateau of 5 min (gradient I) or when optimized, from 20 to 80% acetonitrile in water over 25 min (gradient II). A reequilibration period of 15 min was necessary when TFA was present in the mobile phase. Solvents were filtered through a Millipore filter (0.45 μm) and helium degassed prior to use. Commercial saponins were dissolved in methanol (Omnisolv grade, MCB) at a concentration of 1 mg/ml and centrifuged at 2500 *g* for 3 min to remove any particulate material. 10–30 μl samples were injected. Saponins were eluted at 1 ml/min and detection was monitored at 210 nm.

RESULTS AND DISCUSSION

Comparison of resolution on different RP columns

The resolving power of two bonded-phase packings, C_{18} and cyano which are known to have different retention selectivities, was tested. The saponins when chromatographed individually on either a C_{18} or a cyano column, showed sharp peaks with the exception of β-escin and gypsogenin-3-glucuronide which had elution volumes of about 3 ml and could overlap the elution of other saponins; therefore, these two compounds were excluded when comparing columns. Reequilibration times were 10 min for both columns.

Chromatography of saponins on the C_{18} column using gradient I did not resolve hederacoside C from asiaticoside (Table I). However, by changing to a more

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TABLE I

RETENTION TIMES (min) OF TRITERPENE SAPONINS

Retention times of compounds run individually are enclosed in parentheses. Grad. I: linear gradient from 0 to 80% acetonitrile over 25 min + plateau (5 min). Grad. II: linear gradient from 20 to 80% acetonitrile over 25 min. Grad. II + TFA: gradient II with solvents containing 0.1% TFA.

Saponin	*Cyano column*		*C_{18} column*		
	Grad. I	*Grad. II*	*Grad. I*	*Grad. II*	*Grad. II + TFA*
Madecassoside	13.7	5.2	19.1	12.6	12.6
Asiaticoside	–*	6.4	20.2	14.0	13.6
Hederacoside C	16.4	6.4	20.2	14.7	14.3
Chrysantellin A	18.2	9.8	21.7	16.7	16.0
β-Escin	–	–	–	(12.5)	17.5
Echinocystic acid-3-glucoside	21.5	13.2	25.7	21.8	21.0
α-Hederin	21.5	15.1	26.5	23.0	21.9
Gypsogenin-3-glucuronide	–	–	–	(17.8)	22.7

* Not available at the time of experiment.

hydrophobic system and by decreasing the slope of the gradient (gradient II) baseline resolution was obtained for the six saponins (not including glucuronic acid), and the last eluting compound, α-hederin, eluted more rapidly (Table I). As expected, saponin retention was shorter on the cyano column than on the C_{18} column. By combining the cyano column and gradient II, the more hydrophobic glycoside, α-hederin, eluted in only 15.1 min. While chromatographic runs were faster on the cyano column, some of the saponins co-eluted: echinocystic acid-3-glucoside and α-hederin when using gradient I, and hederacoside C and asiaticoside when using gradient II. By contrast, the C_{18} column and gradient II permitted excellent resolution of standard saponins and gave satisfactory run times, and these conditions were used for further experiments.

Effect of an ion-interaction reagent

Ion-interaction reagents have been used successfully to avoid peak tailing and to modify selectivity in RP systems[10]. Contrary to findings of Domon *et al.*[6], the glucuronides studied here, β-escin and gypsogenin-3-glucuronide, showed extensive band broadening. These saponins contain a glucuronic acid moiety which may ionize in aqueous solution and thereby cause peak broadening. In an attempt to improve peak shape of the glucuronic acid-containing saponins, a known ion-suppression reagent, trifluoroacetic acid[11] was added to the mobile phase. TFA reagent was chosen because of its low UV absorbance that allows sample detection without baseline drift.

When saponins having a glucuronic acid moiety (β-escin and gypsogenin-3-glucuronide) were chromatographed in the presence of TFA-containing solvents, they eluted as sharp peaks. Based on these results, a complete separation of eight triterpene saponins was achieved on the C_{18} column with a gradient from 20 to 80% acetonitrile in water containing 0.1% TFA over 25 min at 1 ml/min flow-rate (Fig. 1). β-Escin

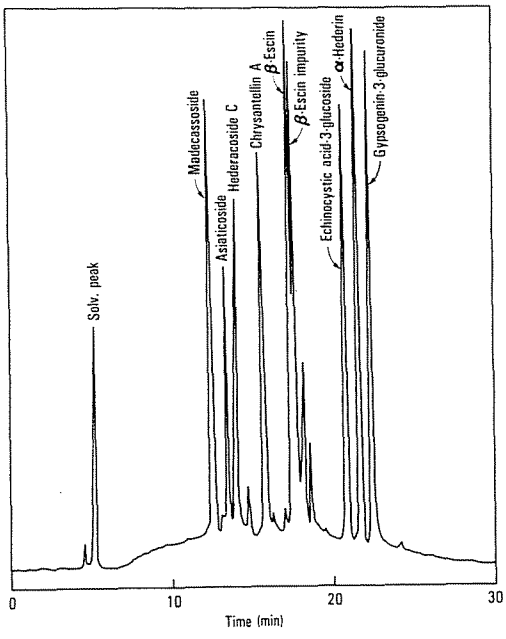


Fig. 1. Chromatogram of a mixture of eight saponins using a C_{18} column eluted with a linear gradient of 20 to 80% acetonitrile in water containing 0.1% TFA over a 25-min period with a flow-rate of 1 ml/min.

and gypsogenin-3-glucuronide were more strongly retained and their retention times increased from 12.5 to 17.5 min and from 17.8 to 22.7 min, respectively. Retention times of all other saponins were only slightly changed by the presence of TFA in the eluent (Table I). Curiously, α-hederin and echinocystic acid-3-glucoside did not show peak broadening, although they contain a carboxyl group in their triterpene moiety. The enhanced retention of such glycosides can be explained by the suppression of ionization of the carboxyl group by TFA, which is a much stronger acid.

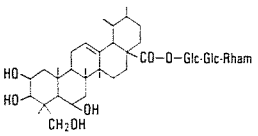
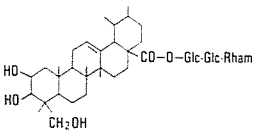
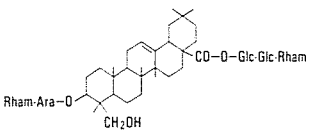
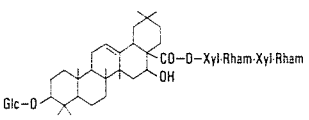
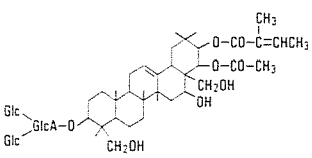
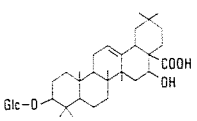
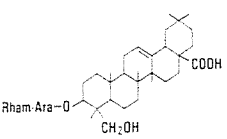
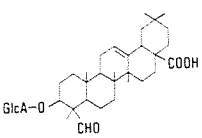
Our data clearly demonstrate the advantages of using TFA reagent for the separation of saponins by RP-HPLC, such as increased column efficiency for acidic compounds and improved resolution of the whole system. In addition, TFA should be suitable for preparative HPLC because it is sufficiently volatile to be removed by freeze-drying. Chromatography with this type of reagent may sometimes require much longer reequilibration times[12], but in this study the addition of TFA to the eluent increased the reequilibration time by only 5 min.

The order of elution of the saponins studied here was a function of the hydrophobicity of the triterpene portion of the molecule, hence the number and position of polar groups and sugar side chains on that moiety (see Table II) were more important in determining retention time than the total number of sugar residues in the side chain. Thus, madecassoside with five aglycone polar groups, eluted ahead of asiaticoside with four; and both eluted sooner than hederacoside C and chrysantellin A with two more sugar residues but only three polar substituents and a single sugar side chain. β-Escin eluted next: obviously the nonpolar tails on its E-ring substituents counteracted the effect of the polar D- and E-ring substituents. Echinocystic acid-

TABLE II

CHEMICAL STRUCTURES OF TRITERPENE SAPONINS

Abbreviations used: Glc, glucose; Rham, rhamnose; Xyl, xylose; Ara, arabinose; GlcA, glucuronic acid.

Saponin	*Structure*	*Aglycone*
Madecassoside:		Madecassic acid
Asiaticoside:		Asiatic acid
Hederacoside C:		Hederagenin
Chrysantellin A:		Echinocystic acid
β-Escin:		Protoescigenin
Echinocystic acid-3-glucoside:		Echinocystic acid
α-Hederin:		Hederagenin
Gypsogenin-3-glucuronide:		Gypsogenin

3-glycoside and α-hederin further illustrate the relative importance of polar *versus* sugar groups, the former with one sugar eluting before the latter with two. Echinocystic acid-3-glucoside elutes first because the effect of its hydroxyl group (which is *trans* with respect to the carboxyl group) is not masked by an adjacent sugar sub-

stituent. In the case of hederacoside C *versus* chrysantellin A the free hydroxyl group is shielded by the presence of a sugar residue in both, but by extending further from the plane of the aglycone molecule the methylene hydroxyl group of hederacoside C is shielded to a lesser extent; hence, it is less hydrophobic and elutes first.

Our results clearly show the effectiveness of RP-HPLC for the separation of triterpene saponins. Modification of the mobile phase by addition of an ion-interaction reagent (TFA) greatly enhanced efficiency and selectivity of the chromatographic system. This method can thus be applied to the separation of saponins and related glycosides.

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